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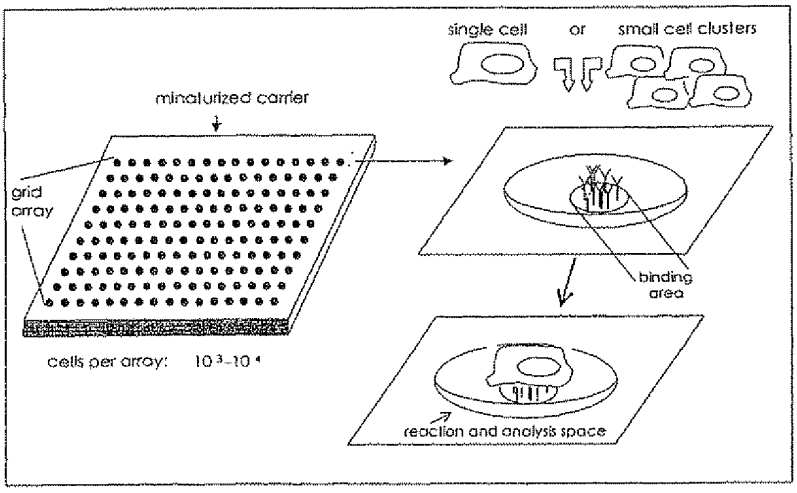

(57) Abstract: The present invention describes a novel method, a specialised device and a kit for quantitative analysis of mutation loads in cells and tissues. The method of the invention consists of analysing mutations, predominantly within microsatellite DNA, in a large number of single-cells or small cell-clusters independently. This approach is particularly suitable when the detection of a specific molecular change is easily obscured by the presence of normal molecules or by the presence of molecular changes with opposite orientation. Furthermore, it enables the concurrent quantification of mutant frequencies as well as of genetic heterogeneity. The specialised device contemplates the immobilisation of a large number of cells in a specified geometric order, primarily as a grid array, onto a miniaturised carrier, which then enables the molecular analysis of single-cells or small cell-clusters in separate reaction and analysis spaces, thus providing information on the number of modified cells and the nature of the molecular alteration.

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A METHOD AND A DEVICE FOR QUANTIFICATION OF MUTATION LOADS

FIELD OF THE INVENTION

The present invention relates to the field of molecular diagnostics. In particular, the invention relates to the quantitative analysis of mutations in cells and tissues, to the identification of the predominant form of genetic instability, and to the screening for genetic susceptibility to malignant tumors.

BACKGROUND OF THE INVENTION

The invention relates to a method, a device and a kit for the quantification of mutation loads in cells and tissues by determining mutant frequencies and genetic heterogeneity on the single-cell or oligo-cell level. Its main application is the detection and quantification of mutations in preneoplastic and neoplastic conditions in order to identify patients at risk for cancer development, to clarify diagnosis of malignant disease, to specify the mechanisms of genetic instability, to quantify the degree of genetic heterogeneity in malignant tumors as well as to screen for the presence of mutations conferring resistance to cancer treatment.

Tumorigenesis and tumor progression can be considered an evolutionary process in which mutant and more tumorigenic subpopulations are sequentially selected and derived from less tumorigenic or benign progenitor cells. This view of cancer first proposed by P.C. Novell in 1976 is supported by genetic

data demonstrating the monoclonal origin of malignant tumors and the multistep nature of tumorigenesis (Fearon & Vogelstein, 1990, Loeb & Christians, 1996, Nowell, 1976, Whittemore & Keller, 1978). The two main driving forces of tumorigenesis are on one hand a positive growth selection which may be mediated by external influences as well as by mutations in genes responsible for cell growth and apoptosis regulation and on the other hand an enhanced mutation rate leading to genetic instability.

Several human diseases are associated with an enhanced mutation rate and a predisposition to cancer. The genetic defects mostly reside in systems responsible for detecting or correcting genetic damage from external or internal sources. Many different genes are involved in genetic instability. Specific genetic alterations could be identified for hereditary conditions like ataxia teleangiectasia (ATM), xeroderma pigmentosum (RPA, ERCC1, ERCC3, ERCC5, ERCC6, XPC), Li-Fraumeni syndrome (P53), familial breast cancers (BRCA1, BRCA2), hereditary nonpolyposis colorectal cancer (MLH1, MSH2, MSH3, MSH6, PMS1, PMS2). Although defects in many different genes may lead to genetic instability, genetic instability by itself may be classified into only two groups, which can be detected by one test.

Defects of the repair of double strand DNA-breaks (Lopes et al., 1999) or of centrosome function (Ghadinmi et al., 2000) and spindle assembly lead to losses or duplications of long stretches of chromosomal DNA (gross chromosomal rearrangements) which can be detected by loss of heterozygosity (LOH) of polymorphic DNA such as microsatellite DNA (Weber & May, 1989).

Defects of genes responsible for the repair of DNA mismatches enhance the occurrence of point mutations, small DNA deletions and frameshift mutations in repetitive DNA sequences such as microsatellite DNA (Ionov et al., 1993, Kolodner & Marsischky, 1999). This kind of genetic instability is named microsatellite instability (MSI).

Therefore, the analysis of microsatellite DNA, which can be easily amplified by the polymerase chain reaction (PCR, US patent 4,683,195) enables the detection of both mechanisms of genetic instability (US patents: 6,191,268; 6,165,713).

Determination of the true mutation rate of prokaryotic and eukaryotic cells generally requires cell culture techniques, which are laborious and time-consuming (Kendal & Frost, 1988). Major technical problems associated with the measurement of mutation rates root in the low mutation rate of eukaryotic cells, which ranges from approx. 10^{-6} - 10^{-7} in normal human cells (Boesen et al., 1995) and 10^{-6} to 10^{-4} (Eshleman et al., 1995, Glaab & Tindall, 1997) in tumor cells. The statistics of rare events require significantly large numbers of samples and repetitions of the mutation assays in order to yield valid data.

For clinical purposes, the exact knowledge of the mutation rate may be replaced by the determination of the frequency of mutant cells in a specific tissue (Green et al., 1995). An enhanced mutant frequency will reflect both, an enhanced mutation rate as well as the positive selection of mutant cells and will, therefore, provide a risk estimate for the development of malignant tumors. It has been demonstrated that mu-

tant frequencies rise with age and show significant difference between individuals (Grist et al., 1992).

A second indicator of an enhanced mutation rate is genetic heterogeneity. It is an indirect evidence of the mutational pressures during tumorigenesis and tumor progression and it reflects the multistep nature of the neoplastic transformation as genetic heterogeneity may represent different steps of the early tumorigenic process. Analysis of genetic heterogeneity can, therefore, be used to estimate mutation rates in malignant and premalignant tissues. Genetic heterogeneity can be observed in many human malignancies (Boni et al. 1998, Fujii H et al. 1996, Mirchandani et al., 1995). It is important to point out that analysis of genetic heterogeneity in primary colon cancers has demonstrated that the benign adenoma, which is the precursor of colon cancer yields a higher degree of genetic heterogeneity than the derived malignant tumor cell clone (Shibata et al. 1993, Boland et al. 1995, Tollenaar et al. 1997). Analysis of genetic heterogeneity may, therefore, be particularly suitable to detect premalignant lesions at risk for malignant conversion.

It has been demonstrated that analysis of microsatellite DNA of small microdissected cell-clusters of malignant tissues enables the identification of the two main mechanisms of genetic instability (Boni et al. 1998, Rübben et al., 2000).

In several cases, analysis of microdissected tissue samples of malignant melanomas showed the concurrent presence of cells with losses of either the maternal or the paternal microsatellite allele (Morita et al., 1998, Rübben et al., 2000). Analysis of pooled tissue from these samples would

have made the demonstration of genetic instability as well as its quantification impossible.

The concurrent presence of cells with gross chromosomal rearrangements that target the same chromosomal region but that lead to loss of one chromosomal region in some cells and loss of the homologue chromosomal region of the other chromosome in other cells is an indirect evidence of the selective pressure for this chromosomal rearrangement and a clear indicator of multilineage progression in these malignant tumors. Multilineage progression could be demonstrated in many human malignancies (Tsao et al., 1999). Therefore, it is highly probable that analysis of genetic instability by PCR-amplification of microsatellite DNA will underestimate mutant frequencies in most human cancers if pooled cells are analysed.

Multilineage progression may also obscure the detection of microsatellite instability, as microsatellite sequences may elongate or contract in tumor cells with defects of DNA mismatch repair genes. Thus, a continuous spectrum of elongated or contracted microsatellite sequences may not be detected in pooled tumor cells.

A second problem associated with the quantification of mutant frequencies and genetic heterogeneity results from the fact that tumor cells are under a strong negative selective pressure under *in-vivo* conditions: The action of the immune system and of cancer treatments as well as the occurrence of mutations in essential genes lead to a constant reduction of genetic heterogeneity within the tumor cell population. Evidence of negative selective pressure during tumor progression

could be demonstrated in malignant melanoma (Rübben et al., 2000). Positive selection of tumor cells through a strong growth advantage basically has the same effect and will reduce overall genetic heterogeneity within the tumor cell population. The biasing effect of selective pressure during tumor progression is stronger when pooled tissue is analysed, as the predominant tumor cell clone will obscure the presence of additional mutated tumor cells.

It has been shown by our laboratories that analysis of multiple tumor cell areas of approximately 200 cells each within malignant and premalignant tissues gives the opportunity to assess mutant frequencies as well as the overall genetic heterogeneity within the analysed specimens (Rübben, 2001). It also has been demonstrated that premalignant conditions can be differentiated from benign tissue by this technique (Rübben, 2001). Nevertheless, analysis of mutant frequencies by microdissection has major limitations:

i. The number of cells can not be reduced to fewer than approx. 50 cells as DNA of microdissected tissue is often degraded by fixation and by the method of microdissection. When only few cells are analysed, amplification of microsatellite DNA may arbitrarily favour one allele over the other which would lead to a false positive LOH result (Sieben NL et al., 2000). On the other hand, the analysis of large cell clusters reduces the sensitivity to detect genetic instability.

ii. Determination of mutant frequencies by analysis of cell clusters of 100 to 200 cells would require screening of a very large number of microdissected tumor cell areas as, depending on the mutation rate, 100 to 10,000

areas per specimen may have to be analysed in order to yield statistically significant results. As microdissection analysis is extremely laborious, analysis of mutant frequencies by microdissection of 100 to 10,000 areas using conventional techniques is not practical for routine applications.

Multilineage progression in malignant and premalignant tissues is the reason why mutation detection using microsatellite analysis is greatly impaired when tissue specimens are analysed as a whole. Furthermore, as mutations represent rare events, a large number of cells or cell-clusters have to be screened in order to yield statistically significant results. The currently available techniques are too labour-intensive to routinely estimate mutant frequencies and genetic heterogeneity in clinical specimens through the analysis of microsatellite DNA.

For the reasons set out above an estimation of mutant frequencies and genetic heterogeneity using microsatellite analysis does require separate analysis of isolated single-cells or of small cell-clusters in order to detect the majority of the present mutations. As mutations represent rare events, multiple cells or cell-clusters have to be screened in order to yield statistically significant results.

The present invention provides both a rapid and easy method and device to perform analysis of mutant frequencies and genetic heterogeneity in multiple single-cells or small cell-clusters.

SUMMARY OF THE INVENTION

The proportion of mutant cells (mutant frequency) as well as the degree of genetic heterogeneity in premalignant and malignant cells or tissues constitute the mutation load. The mutation load is dependent on the generation number of the clonal expansion as well as on the degree of the underlying genetic instability. A high mutation load is a risk factor for neoplastic transformation (DePinho, 2000), it may define the time point of neoplastic transformation, it is an indicator of tumor progression and it is a negative prognostic factor of tumor therapy.

The present invention has resulted from the finding that analysis of mutant frequencies and genetic heterogeneity within premalignant and malignant tissues and cells requires the separate analysis of a large number of cells on the single-cell or oligo-cells level.

It has been shown that multilineage progression is a common feature of malignant melanoma (Rübben et al., 2000). Multilineage progression as well as strong selection for gross chromosomal rearrangements targeting the same chromosomal region but resulting in loss of one chromosomal region in some cells, and loss of the homologue chromosomal region on the other chromosome in other cells, greatly limit the application of microsatellite analysis for the quantification of mutant frequencies and genetic heterogeneity in premalignant and malignant cells or tissues.

Multilineage progression may also obscure the detection of microsatellite instability, as microsatellite sequences may elongate or contract in tumor cells with defects of DNA mismatch repair genes. Thus, a continuous spectrum of elongated or contracted microsatellite sequences may not be detected in pooled tumor cells.

Quantification of mutant frequencies and genetic heterogeneity may also be biased by selective pressures within the tumor cell microenvironment. A strong negative selective pressure which reduces mutant frequencies and genetic heterogeneity may result under *in-vivo* conditions through the action of the immune system and by cancer treatment. Furthermore, the occurrence of mutations in essential genes may also lead to a constant reduction of genetic heterogeneity within the tumor cell population. Positive selection of tumor cells through a strong growth advantage has the same effect on overall genetic heterogeneity within the tumor cell population but will result in an overestimation of mutant frequencies. We could demonstrate evidence of negative as well as positive selective pressures during tumor progression in malignant melanoma, but it is conceivable that strong selective pressure is present in most human cancers (Rübben et al., 2000). The biasing effect of selective pressure during tumor progression is stronger when pooled tissue is analysed as the predominant tumor cell clone will obscure the presence of additional mutated tumor cells.

Therefore, the main object of the present invention is a method providing an independent mutation analysis of multiple single-cells or small cell-clusters.

Microsatellite DNA is a prime target for mutation analysis as both forms of genetic instability, i.e. gross chromosomal rearrangements and microsatellite instability may be detected by PCR-amplification and analysis of microsatellite DNA sequences. Nevertheless, the principle of the invention may also be applied to other molecular techniques detecting loss of heterozygosity or mutations in mismatch repair genes such as analysis of single nucleotide polymorphisms (SNPs) or screening for mutations at repetitive DNA sequences within genomic DNA other than microsatellite DNA. Disease specific mutations, such as codon 12 K-ras-mutations in colorectal cancer, may also represent targets for mutation analysis.

Independent molecular analysis of single-cells or small cell-clusters may, in its most simple form, be performed through limiting dilution of the solution containing purified single-cells. A dilution of approximately 1-2 intact cells per sample is suitable for this quantitative approach.

However, a far more precise and easier to handle technique for the analysis of multiple single-cells or small cell-clusters is the analysis by cell-microarray-chips (CMC).

Therefore, it is a further important object of the present invention to provide a specialized CMC for the analysis of mutant frequencies and genetic heterogeneity by immobilising a large number of cells in a specified geometric order, primarily as a grid array, onto a miniaturised carrier which then enables the molecular analysis of single-cells or small cell-clusters in separate reaction and analysis spaces, thus

providing information on the number of modified cells and the nature of the molecular alteration.

Selective analysis of specific cells is ensured by interaction of the binding area on the carrier with target molecules on the surface of these cells. The number of cells bound to each analysis area is determined by the surface area of the molecules mediating specific binding on the carrier. Antibodies, integrins, lectins, receptor-ligand complexes, bioengineered molecules or related mechanisms and the like, may enable binding.

Molecular analysis can be performed as described above and may be done directly on the carrier or after transfer of material onto a specific analysis device.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Analysis of genetic instability at microsatellite marker D18S65 in three microdissected regions of a malignant melanoma (electropherogram analysis, ABI PRISM 373, molecular size marker: GENESCAN 400HD ROX, PE Applied Biosystems). Area 1 shows loss of heterozygosity (LOH) by the absence of allele A. Area 2 demonstrates microsatellite instability (MSI) as allele A is shifted to the right indicating an expansion of DNA repeat sequences. Area 3 does not demonstrate LOH or MSI.

Figure 2: Analysis of genetic instability at microsatellite marker D9S162 in eight microdissected regions of a dysplastic melanocytic nevus (electropherogram analysis, ABI PRISM 373, molecular size marker: GENESCAN 400HD ROX). Areas 1, 2 and 7 show LOH by the absence of allele B. Area 4 demonstrates partial LOH by the absence of allele A as well as MSI as allele B is shifted to the left indicating a contraction of DNA repeat sequences. Areas 3, 5, 6 and 8 also show MSI. N represents the normal tissue of the patient without LOH or MSI.

Figure 3: Graphic display of LOH-mutation index found in 5 normal and 5 dysplastic melanocytic nevi.

Figure 4: Analysis of genetic instability at marker D18S65 in tumor cell line MEL-IM (electropherogram analysis, ABI PRISM 373, molecular size marker: GENESCAN 400HD ROX). Data were generated by performing 100 PCR reactions containing 1-2 tumor cells per reaction.

Figures 5-7: Schematic drawing of method for mutation detection and quantification of large number of single-cells or small cell-clusters using cell-microarray-chips.

Figure 8: Schematic drawing of a cell-microarray-chip for risk-assessment of melanocytic nevi.

DETAILED DESCRIPTION OF THE INVENTION

The proportion of mutant cells (mutant frequency) as well the degree of genetic heterogeneity in premalignant and malignant cells or tissues reflect the number of mutations which arise during the clonal expansion of a cell lineage. This accumulation of mutations as well as the accumulation of mutant cells constitute the mutation load which is dependent on the generation number of the clonal expansion as well as on the degree of the underlying genetic instability. It has been demonstrated that a high mutation load is a risk factor for neoplastic transformation (DePinho, 2000). The mutation load may define both, the time point of neoplastic transformation as well as the stage of tumor progression. A high mutation load may increase the possibility that resistant cells are already present before a specific anti-tumor therapy is started. Therefore, the mutation load may be regarded as a general negative prognostic factor of any anti-tumor therapy.

The present invention is a result from the recent finding that quantification of mutant frequencies and genetic heterogeneity within premalignant and malignant tissues and cells does require the analysis of a large number of cells on the single-cell or oligo-cells level:

Firstly, it has been confirmed that multilineage progression is a feature of malignant melanoma by quantitative analysis of mutations affecting microsatellite DNA (LOH and MSI) at different progression stages of a malignant melanoma (Rübben et al., 2000). Multilineage progression had already been shown in other human cancers (Tsao et al. 1999) but the present data demonstrated that multilineage progression results from the repeated occurrence of mutations targeting the same chromosomal regions which reflects strong selective pressure for specific chromosomal rearrangements in malignant tumors. As these mutations occur independently, one result of multilineage progression is that some cells may harbour loss of one allele located in a specific chromosomal region whereas other cells of the same tumor may yield loss of the homologue chromosomal region on the other chromosome.

This let to the conclusion that when pooled cells of a tumor or a preneoplastic tissue are analysed, the presence of cells harbouring allele losses of the same chromosomal region, but in some cells affecting one allele and in other cells affecting the homologue allele on the other chromosome, may result in a decreased detection sensitivity of this class of mutations.

The inventor has then analysed mutations of microsatellite DNA in multiple microdissected areas of primary malignant melanomas and has been able to demonstrate a very high degree of genetic heterogeneity with different tumor cell clones displaying loss of heterozygosity (LOH) of either alleles.

Mutation loads in these tumors may not be correctly quantified if pooled tumor cells are taken for analysis. It also has been demonstrated that a primary malignant melanoma may harbour both classes of genetic instability in different tumor areas, i.e. gross chromosomal rearrangements detected by LOH at microsatellite loci as well as microsatellite instability (Rübben et al., 2000). Figure 1 shows an example. Furthermore, as microsatellite sequences may elongate or contract in tumor cells with defects of DNA mismatch repair genes, a continuous spectrum of elongated or contracted microsatellite sequences may also not be detected in pooled tumor cells. Therefore, multilineage progression may obscure the detection of microsatellite instability as well.

Furthermore, it has been found that mutant frequencies and genetic heterogeneity may also be biased by selective pressures within the tumor cell microenvironment. A strong negative selective pressure which reduces mutant frequencies and genetic heterogeneity may be the consequence of the action of the immune system and or of a specific cancer treatment. Furthermore, the occurrence of mutations in essential genes may also lead to a constant reduction of genetic heterogeneity within the tumor cell population. Positive selection of tumor cells through a strong growth advantage has the same suppressing effect on overall genetic heterogeneity within the tumor cell population, but will result in an increase of mutant frequencies. Evidenvce of negative as well as positive selective pressures during tumor progression in malignant melanoma (Rübben et al., 2000) has been demonstrated, but it

is conceivable that strong selective pressure is present in most human cancers.

The biasing effect of selective pressure during tumor progression is stronger when pooled tissue is analysed as the predominant tumor cell clone will obscure the presence of additional mutated tumor cells.

When it became obvious that mutations resulting in loss of heterozygosity or microsatellite instability may be best detected by analysing multiple microdissected cell-clusters, analysis of multiple cell-clusters of the same tissue has been carried out enabling the quantitative measurements of mutation loads: Five normal melanocytic nevi as well as five dysplastic melanocytic nevi were taken, which represent a preneoplastic condition for the development of malignant melanoma and have been analysed for the presence of LOH or microsatellite instability. Mutant frequencies (m) as well as genetic heterogeneity (= frequency of distinguishable cell clones = n) were combined to a mutation index by the formula: $\sqrt{m \times n}$. Mean LOH-mutation index was 0.17 in normal melanocytic nevi but 0.48 in dysplastic melanocytic nevi ($p = 0.004$). Thus, the preneoplastic condition for malignant melanoma displayed a significantly higher mutation load than the benign melanocytic nevus. Figure 2 shows a typical example of microsatellite analysis of a dysplastic melanocytic nevus while figure 3 displays the results of the study graphically.

As a result of these data, it was reasoned that the most effective method to quantify mutation loads is the analysis of

multiple cells on the single-cell or oligo-cells level. Therefore, a limiting dilution of the melanoma tumor cell line MEL-IM was performed in order to obtain a dilution of approximately 1-2 intact cells per PCR reaction. Analysis of 100 cells at microsatellite marker D18S65 showed that 53% of all cells demonstrated LOH of the allele of smaller molecular size, 14% showed LOH of the allele of higher molecular size, while 33% of cells did not display gross chromosomal rearrangements at this marker (Fig. 4). None of these tumor cells demonstrated microsatellite instability. Analysis of 100 cultured primary human melanocytes did not demonstrate LOH or MSI in any of these cells. These data confirmed that analysis of microsatellite DNA of multiple cells on the single-cell or oligo-cells level yields quantitative data on mutant frequencies, on genetic heterogeneity as well as on the predominant class of genetic instability present in tumor cells.

Therefore, the preferred embodiment of the invention consists of a separate mutation analysis of multiple single-cells or small cell-clusters. The method of diagnosing comprises the steps of:

1) Obtaining a solution of single-cells or small cell-clusters of premalignant or tumor mammalian tissue;

2) Enriching tumor- or tissue-specific cells, if necessary;

3) Obtaining normal mammalian tissue of the same organism;

4) Extracting DNA or RNA from a large number of single-cells or small cell-clusters of the premalignant or tumor tissue in separate and small scale reaction volumes;

5) Extracting DNA or RNA from pooled normal mammalian cells or tissue;

6) Independent PCR, reverse transcription PCR (RT-PCR) or DNA-DNA/RNA-DNA/RNA-RNA-hybridisation performed in small scale volumes on all extracted DNA or RNA samples in order to detect microsatellite instability, gross chromosomal rearrangements or disease specific mutations and changes in gene expression;

7) Performing independent analysis of PCR- or RT-PCR-products or DNA-DNA/RNA-DNA/RNA-RNA-hybrids of each analysed single-cell or small cell-cluster as well as of the normal DNA/RNA sample;

8) Comparing the molecular results obtained from preneoplastic or tumor tissue with data generated from the normal DNA/RNA sample; and

9) Determining the nature of genetic instability and quantifying the frequency of specific mutants as well as the degree of genetic heterogeneity in the analysed premalignant or tumor tissue.

Although fresh and archival tissue may be used for analysis, best results are obtained with unfixed cells with intact genomic DNA. Blood cells, cells of effusions or smears may be analysed directly.

Solid tissues are analysed after enzymatic or mechanical dissociation of the tissue into single-cells or small cell-clusters.

Microdissection of formalin-fixed tissue sections of routine histology slides at multiple regions may be used to analyse archival formalin-fixed tissue specimen that may not be transformed into a single-cell solution due to crosslinking of cells. In this case, the number of analysed cells per reaction has to be sufficiently large in order to avoid false positive results through unbalanced degradation of the analysed alleles.

In one embodiment of the present invention, the target for mutation analysis is dependent on the expected frequency of mutant in the analysed samples: Chromosomal regions, which are preferentially mutated (= positively selected mutations) are used for analysis of preneoplastic lesions whereas chromosomal regions that are not preferentially mutated represent targets, which are more suitable for analysis of advanced malignant tumors.

In a preferred embodiment of the invention specific markers are employed. The analysis of melanocytic nevi (moles) and early melanomas is performed by analysis of microsatellite markers on the short arm of chromosome 9 and the long arm of chromosome 10 and 14 such as D9S259, D9S171, D9S942, D9S1748, D9S162, D10S215, D10S209, D14S267 and D14S53. Markers on the short arm of chromosome 18 such as D18S65 are suitable for analysis of mutation loads in advanced malignant melanomas. Among others, microsatellite markers D6S260, D6S261, D9S162 and D18S65 are particularly suitable for the analysis of cu-

taneous T-cell lymphomas. Markers on the short arm of chromosome 3 such as D3S1300, D3S4103 and D3S1283 are used for the analysis of cervical scrapes, cervical cancer as well as epithelial head and neck cancers. Analysis of sputum for lung cancer screening can be performed with markers on 9p and 9q as well as with microsatellites on the short arm of chromosome 8 such as D8S133 and D8S137.

An alternative system for demonstration of gross chromosomal rearrangements would be the detection of loss of heterozygosity by analysis of single nucleotide polymorphisms (SNPs). Allele specific PCR amplification or hybridisation with sequence specific DNA or RNA probes labelled with enzymes or fluorescent dyes can be used to discriminate between alleles. Measurement of melting curves of DNA-DNA or RNA-RNA or DNA-RNA-complexes by laser scanning represents an alternative method to distinguish between alleles.

Competitive multiplex PCR may also be used to detect gross chromosomal rearrangements leading to homozygous loss of tumor suppressor genes such as $p16^{ink4}$. Loss of chromosomal material can also be demonstrated by hybridization with specific DNA or RNA probes.

Analysis for mutations at repetitive DNA sequences within genomic DNA other than microsatellite DNA may also be performed in order to detect defects of DNA mismatch repair.

Disease specific mutations, such as codon 12 K-ras-mutations in colorectal cancer, may also represent molecular targets for determination of mutation loads. They may be demonstrated by sequence-specific polymerase chain reaction (SSP-PCR) or by hybridisation with sequence-specific DNA probes labelled with enzymes or fluorescent dyes.

Defects of RNA transcription due to specific mutations may be detected after reverse transcription and PCR (RT-PCR) with specific primers.

In another embodiment of the invention, mutation loads are analysed by determining mutant frequencies and the frequency of individual cell clones (genetic heterogeneity) separately.

In yet another embodiment, mutation loads are determined by a mutation index integrating mutant frequencies (m) and the frequency of individual cell clones (n) by the formula: $\sqrt{mxn}$.

In another preferred embodiment, the identification of individual cell clones is enhanced by analysing a set of multiple microsatellite markers on each single-cell or on a small cell-cluster. This procedure enhances the probability of detecting different cell clones by the pattern of chromosomal rearrangement or by the presence of mutated alleles.

Primary fields of application of the present invention are the detection and quantification of mutations:

- in preneoplastic conditions in order to identify patients at risk for cancer development;
- in order to clarify diagnosis and progression state of malignant disease;
- in order to specify the mechanisms of genetic instability and the degree of genetic heterogeneity in malignant tumors; and
- in order to screen for the presence of mutations conferring resistance to cancer treatment.

It is the most preferred embodiment of the present invention to employ cell-microarray-chips (CMC) for the analysis of multiple single-cells or small cell-clusters.

By employing CMC for the analysis of mutant frequencies and genetic heterogeneity according to the present invention a large number of cells are immobilised in a specified geometric order, primarily as a grid array, onto a miniaturised carrier which then enables the independent analysis of single-cells or small cell-clusters in separate reaction and analysis spaces (Fig. 5). Said approach provides information on the number of modified cells as well as on the nature of the molecular alteration.

In a further embodiment of the invention solid tissues are analysed after enzymatic or mechanical dissociation of tissue into a single-cell suspension while blood cells, cells of effusions or smears may be analysed directly as single-cell suspensions. The single-cell suspension then interacts with a carrier that bears multiple binding areas for target cells present within the cell suspension (Fig. 6).

In a particularly preferred embodiment, the binding areas on the carrier are organised in a defined geometric order that may form a two dimensional grid array, a more complex two dimensional array or even a three dimensional organisation depending on the requirements of the subsequent analysis procedure. The binding areas define separate reaction and analysis spaces. The number of individual analysis spaces depends on the scope of the diagnostic procedure. The analysis spaces are densely packed. The carrier bearing the binding areas forms a miniaturised device in order to enable simultaneous analysis of multiple single-cells or multiple small cell-clusters. The preferred number of analysis spaces lies in the range of 50 to 20000.

In another preferred embodiment, selective analysis of specific cells is ensured by interaction of the binding area on the carrier with target molecules on the surface of these cells. Among others, antibodies, integrins, lectins, receptor-ligand complexes, bioengineered molecules or related mechanisms, enable binding (Fig. 6).

In yet another embodiment, the number of cells bound to each analysis area is determined by the surface area of the molecules mediating specific binding on the carrier (Fig. 6). This approach enables to modify the number of cells that are analysed together in one analysis space according to requirements of the inventive method.

Procedures related to the analysis of the bound cells, like staining or incubation with antibodies, are performed directly on the carrier. Molecular detection procedures are performed after providing separate reaction volumes filled with reaction and detection reagents. In one embodiment, separate small scale reaction volumes are provided by a cover yielding concave depressions. The carrier may bear concave depressions as well (Fig. 7).

Molecular analysis may be performed using antibody-antigen binding, DNA or RNA hybridisation, polymerase chain reaction (PCR) or computer-assisted image processing of specific morphological changes. Analysis may be performed directly on the carrier or after transfer of material onto a specific analysis device.

In a further embodiment of the invention, for molecular analysis on the miniaturised carrier/cover system fluorescent probes are used that can be detected by laser scanning through a transparent cover (Fig. 8). Simultaneous analysis of melting or dissociation curves may provide information on base composition and the presence of mutation within the ana-

lysed DNA or RNA molecules. Alternatively, material from the separate reaction and analysis spaces can be transferred to an external analysis device after piercing of a thin membrane on the outer surface of the cover (Fig. 8).

EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Determination of mutation loads by analysis of multiple single cells

a) Preparation of tissue specimen:

In order to assess mutation load of a melanocytic nevus in a patient with multiple moles, a transverse 0.2 cm thick tissue strip is obtained from the freshly excised nevus and cut into 0.1 x 0.1 cm cubes and placed in vortex at 37°C for 60 min in 1 ml of trypsin/EDTA-solution (Sigma) containing 10 mg/ml dispase (Sigma). The cells are then pelleted through centrifugation at 500 rpm for 3 min at RT, washed with phosphate-buffered saline (PBS) and then resuspended in PBS. The solution may be used directly with a cell-microarray-chip. If analysis is performed by limiting dilution, then concentration of

cells is first determined by cell count and the solution is diluted with PBS to 1-2 cells per µl.

b) Amplification of microsatellite DNA:

Markers D9S259, D9S171 and D9S162 labelled 5'prime with the dyes 6FAM, HEX and NED (Microsynth) are suitable for analysis of mutation load of melanocytic nevi. PCR is done in a total volume of 20 µl with 0.5 U Taq-polymerase (AmpliTaq Gold, PE Applied Biosystems, Foster City). 1 µl of the diluted cell solution is used for every PCR amplification. Final primer concentration is 1.5 µM and Mg^{2+} concentration is 2 mM. 384 samples are prepared on a 384-well microtiter plate with each primer for every analysed nevus. PCR is done on a DNA Thermal Cycler Dual 384-Well GeneAmp® PCR System 9700 (PE Applied Biosystems) using the following temperature profiles: 8 min at 95°C; 2 cycles of 1 min at 58°C, 15 sec at 72 °C, 30 sec at 94.5 °C; 5 cycles of 1 min at 55°C, 15 sec at 72 °C, 30 sec at 94 °C, 22 cycles of 1 min at 55°C, 20 sec at 72 °C, 20 sec at 92 °C; a final step of 25 sec at 55°C and 5 min at 72 °C.

c) Analysis of amplified microsatellite DNA:

1 µl of every PCR amplification sample is mixed with 12 µl of DI Formamide (Sigma) and 0.5 µl of size standard GENESCAN 400HD ROX (PE Applied Biosystems). This mixture is automatically analysed by an automated DNA sequencer (ABI PRISM 3100, PE Applied Biosystems). LOH is scored positive when the fluorescence peak of one allele is reduced in intensity by more than 80 % as compared to the other microsatellite allele.

EXAMPLE 2

Cell microarray chip (CMC) for the analysis of mutation loads in melanocytic nevi

Construction of a CMC is described in figure 8. The carrier consists of a Fast™ slide (Schleicher & Schuell). The array contains 50 to 20'000, preferably 1000 analysis spaces. Each binding area contains a spot of 50 µm diameter that contains 10 nl of biotinylated mouse IgG antibodies (50 µg/l) against the S100 protein expressed predominantly on melanocytic cells. One cell can be bound in each analysis space. The analysis and reaction volume is approximately 0.01 µl. When analysing small cell-clusters 2 to 1000 cells may be immobilized in each space. The reaction volume will increase accordingly. A silicone coating ensures tightness between carrier and cover. The cover yields a 2 µm thick transparent polyester film that can be pierced by a capillary in order to take up the reaction fluid.

EXAMPLE 3

Use of the CMV for determination of mutation loads

The carrier is covered and incubated with the non-diluted single-cell solution at 37 °C for 15 min. Then, the carrier is washed twice with PBS. The carrier is covered with the PCR reagents containing primers for microsatellite markers and hot-start Taq-polymerase. The CMC is placed on a thermal cycler for PCR. The initial denaturation step is performed for 15 minutes at 95°C in order to disrupt the cells and to activate enzyme. PCR amplification is done as described by exam-

ple 1b. After PCR-amplification, fluid is taken from every reaction space with a glass capillary which pierces through the polyester film. This DNA solution is analysed with an automated DNA-sequencer as described by example 1c.

REFERENCES

Boesen JJ, Niericker MJ, Dieteren N, Simons JW: How variable is a spontaneous mutation rate in cultured mammalian cells? Mutat. Res. 1994; 307:121-129.

Boland CR, Sato J, Appelman HD, Bresalier RS, Feinberg AP: Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. Nat. Med. 1995; 1:902-909.

Boni R, Matt D, Voetmeyer A, Burg G, Zhuang Z: Chromosomal allele loss in primary cutaneous melanoma is heterogeneous and correlates with proliferation. J. Invest. Dermatol. 1998; 110:215-217.

DePinho RA: The age of cancer. Nature 2000, 408:248-254.

Eshleman JR, Lang EZ, Bowerfind GK, Parsons R, Vogelstein B, Willson JK, Veigl ML, Sedwick WD, Markowitz SD: Increased mutation rate at the hprt locus accompanies microsatellite instability in colon cancer. Oncogene 1995; 10:33-37.

Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990; 61:759-767.

Fujii H, Marsh C, Cairns P, Sidransky D, Gabrielson E: Genetic divergence in the clonal evolution of breast cancer. Cancer Res. 1996;56:1493-1497.

Ghadimi BM, Sackett DL, Difilippantonio MJ, Schrock E, Neumann T, Jauho A, Auer G, Ried T: Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. Genes Chromosomes Cancer 2000; 27:183-190.

Glaab WE, Tindall KR: Mutation rate at the hprt locus in cancer cell lines with specific mismatch repair-gene defects. Carcinogenesis 1997; 18:1-8.

Green MH, O'Neill JP, Cole J: Suggestions concerning the relationship between mutant frequency and mutation rate at the hprt locus in human peripheral T-lymphocytes. Mutat. Res. 1995; 334:323-339.

Grist SA, McCarron M, Kutlaca A, Turner DR, Morley AA: In vivo human somatic mutation: frequency and spectrum with age. Mutat. Res. 1992; 266:189-196.

Hussain SP, Raja K, Amstad PA, Sawyer M, Trudel LJ, Wogan GN, Hofseth LJ, Shields PG, Billiar TR, Trautwein: Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis oxyradical overload diseases. Proc. Natl. Acad. Sci. 2000; 97:12770-12775.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 1993; 363:558-561.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992; 258:818-821.

Kendal WS, Frost P: Pitfalls and practice of Luria-Delbrück fluctuation analysis: a review. Cancer Res. 1988; 48:1060-1065.

Kolodner RD, Marsischky GT: Eukaryotic DNA mismatch repair. Curr. Opin. Genet. Dev. 1999; 9:89-96.

Loeb LA, Christians FC: Multiple mutations in human cancers. Mutat. Res. 1996; 350:279-286.

Lopes J, Tardieu S, Silander K, Blair I, Vandenberghe A, Palau F, Ruberg M, Brice A, LeGuern E. Homologous DNA exchanges in humans can be explained by the yeast double-strand break pair model: a study of 17p11.2 rearrangements associated with CMT1A and HNPP. Hum. Mol. Genet. 1999; 8:2285-2292.

Mirchandani D, Zheng J, Miller GJ, Gosh AK, Shibata DK, Cote RJ, Roy-Burman P: Heterogeneity in intratumor distribution of p53 mutations in human prostate cancer. Am. J. Pathol. 1995;147:92-101.

Morita R, Fujimoto A, Hatta N, Takehara K, Takata M: Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression. J. Invest. Dermatol. 1998; 111:919-924.

Nowell PC: The clonal evolution of tumor cell populations. Science 1976; 194:23-28.

Rübben A, Babilas P, Baron JM, Hofheinz A, Neis M, Sels F, Sporkert M: Analysis of tumor cell evolution in a melanoma:

evidence of mutational and selective pressure for loss of p16ink4 and for microsatellite instability. J. Invest. Dermatol. 2000; 114:14-20.

Rübben A: Analysis of LOH (9p, 14q)-mutant fractions in dysplastic melanocytic nevi and malignant melanomas reveals genetic instability as driving force of melanoma genesis. Arch. Dermatol. Res. 2001; 293:95.

Shibata D, Schaeffer J, Li Z-H, Capella G, Perucho M.: Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not in adenocarcinomas. J Natl Cancer Inst 1993; 85:1058-1063.

Sieben NL, ter Haar NT, Cornelisse CJ, Fleuren GJ, Cleton-Jansen AM. PCR artifacts in LOH and MSI analysis of microdissected tumor cells. Hum Pathol 2000; 31:1414-1419.

Tollenaar RA, Bonsing BA, Kuipers-Dijkshoorn NJ, Hermans J, van de Velde CJ, Cornelisse CJ, Fleuren GJ: Evidence of clonal divergence in colorectal carcinoma. Cancer 1997; 79:1304-1314.

Tsao JL, Tavare S, Salovaara R, Jass JR, Aaltonen LA, Shibata D: Colorectal adenoma and cancer divergence. Evidence of multilineage progression. Am. J. Pathol. 1999; 154:1815-1824.

Weber JL, May PE: Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. 1989; 44:388-396.

Whittemore A, Keller JB: Quantitative theories of carcinogenesis. SIAM Rev. 1978; 20:1-30.

CLAIMS

1. A method for quantifying mutation loads in cells and tissues by determining mutant frequencies and genetic heterogeneity characterized in that single cells or oligo-cells are analysed separately.

2. The method according to claim 1, said method comprising the following steps of:

(i) obtaining a solution of single-cells or small cell-clusters of mammalian premalignant or tumor tissue;

(ii) enriching tumor- or tissue-specific cells, if necessary;

(iii) obtaining normal mammalian tissue of the same organism;

(iv) extracting DNA or RNA from a large number of single-cells or small cell-clusters of the premalignant or tumor tissue in separate and small scale reaction volumes.

(v) extracting DNA or RNA from pooled normal cells or tissue;

(vi) performing independent PCR, RT-PCR or DNA-DNA/RNA-DNA/RNA-RNA-hybridisation in small scale volumes on the extracted DNA or RNA samples from steps (iv) and (v);

(vii) performing independent analysis of PCR- or RT-PCR-products or DNA-DNA/RNA-DNA/RNA-RNA-hybrids of step (vi);

(viii) comparing the molecular results obtained from step vii;

(ix) determining the nature of genetic instability and quantifying the frequency of specific mutants as well as the degree of genetic heterogeneity in the premalignant or tumor tissue.

3. The method according to claims 1 and 2, wherein the analysed cells are of human or animal origin.

4. The method according to any of claims 1 to 3, wherein the analysed cells are harvested as single-cells, obtained from solid tissues by mechanical or enzymatic dissociation into a single-cell suspension or are obtained from archival formalin-fixed tissue sections.

5. The method according to any of the preceeding claims, wherein chromosomal regions, repetetive DNAs or genes of said cells are analysed.

6. The method according to claim 5, wherein the regions analysed for premalignant lesions are preferentially mutated chromosomal regions, repetetive DNAs or genes.

7. The method according to any of the preceeding claims, wherein the analysed cells originate from melanocytic nevi (moles), melanomas, cervical scrapes and cancer, cutaneous lymphomas as well as sputum.

8. The method according to any of claims 5 to 7, wherein the repetetive DNAs are microsatellite markers.

9. The method according to any of claims 5 to 8, wherein the microsatellite markers are selected from the group consisting of D3S1300, D3S4103, D3S1283, D6S260, D6S261, D8S133, D8S137, D9S259, D9S171, D9S942, D9S1748, D9S162, D10S215, D10S209, D14S267, D14S53, D18S65.

10. The method according to claims 8 to 9, wherein a set of multiple microsatellite markers is used for analysing each single-cell or small cell-cluster.

11. The method according to claims 1-7, wherein the analysed DNAs contain single nucleotide polymorphisms or disease specific mutations.

12. The method according to claims 1-7, wherein changes of gene expression are detected as indicator of disease specific mutations.

13. The method according to any of the preceeding claims, wherein mutation loads are analysed by determining mutant frequencies and/or the frequency of individual cell clones (genetic heterogeneity).

14. A device for quantifying mutation loads in cells and tissues by determining mutant frequencies and genetic heterogeneity characterized in that single cells or small cell clusters are analysed independently on said device.

15. The device according to claim 14, being a cell-microarray-chip (CMC).

16. The device according to claim 14 or 15, comprising

(i) cell surface molecules for a cell-type specific immobilisation of single-cells or small cell-clusters on the carrier;

(ii) immobilized single-cells or small cell-clusters in a specific geometric order;

(iii) a miniaturized carrier carrying the single cells or cell cluster of (ii);

(vi) separate analysis spaces through the form of the carrier and a corresponding cover.

17. The device of claims 14 to 16, wherein cell-clusters of 2-1000 cells are immobilised in each analysis space.

18. The device of any of claims 14 to 17, wherein single-cells or cell-clusters are immobilised in a two-dimensional grid array or a more complex two-dimensional array.

19. The device of claims 14 to 17, wherein single-cells or cell-clusters are immobilised in a three dimensional geometric order.

20. The device according to claims 14 to 17, wherein single-cells or cell-clusters are immobilized in 50 to more than 20.000 individual analysis spaces.

21. The device according to claims 14 to 16, wherein the surface molecules on the cells are proteins or glycoproteins and the molecules on the carrier are specific mono- or polyclonal antibodies or engineered recombinant molecules.

22. The device according to claim 21, wherein the surface molecules on the cells are receptors or ligands and the molecules on the carrier are the corresponding natural or bioengineered ligands or receptors.

23. The device of claims 21 and 22, wherein the surface molecules on the cells are integrins and the molecules on the carrier are the corresponding glycoproteins or extracellular matrix proteins.

24. The device of any of the preceeding claims, wherein the analysis of the bound cells is performed by molecular techniques detecting DNA or RNA.

25. The device of claim 24, wherein the analysis of the bound cells is performed by polymerase chain reaction (PCR).

26. The device according to claim 25, wherein the analysis of the bound cells is performed by molecular techniques using PCR-amplification of microsatellite DNA.

27. The device of claims 26, wherein the analysis of the bound cells is performed by molecular techniques using the PCR-amplification of DNA sequences with single nucleotide polymorphisms (SNPs).

28. The device according to any of the claims 14 to 28, wherein the analysis of the bound cells is performed by sequence-specific polymerase chain reaction (SSP-PCR) for detection of disease specific mutations.

29. The device according to any of the claims 14 to 28, wherein the analysis of the bound cells is performed by hybridisation of fluorescent DNA or RNA probes.

30. The device of claim 14, wherein the analysis of the bound cells is performed by molecular techniques analysing

sequence specific melting curves of DNA-DNA or RNA-RNA or DNA-RNA-complexes.

31. The device according to any of the preceeding claims 14 to 30, wherein the analysis of the bound cells is performed directly on the carrier/cover system.

32. The device according to any of the preceeding claims 14 to 31, wherein the analysis of the bound cells is performed without physical contact with the cells or the surrounding medium by laser scanning.

33. The device of claim 13, wherein the analysis of the bound cells is performed after creating a physical contact with the cells or the surrounding medium.

34. The device according to any of the claims 14 to 30, wherein the analysis of the bound cells is performed by molecular techniques requiring multiple steps, some steps being performed on the carrier/cover system and some steps are performed after transfer of material to an external analysis system.

35. A kit comprising the device of claim 14 for performing the method of claim 1.

Figure 1.

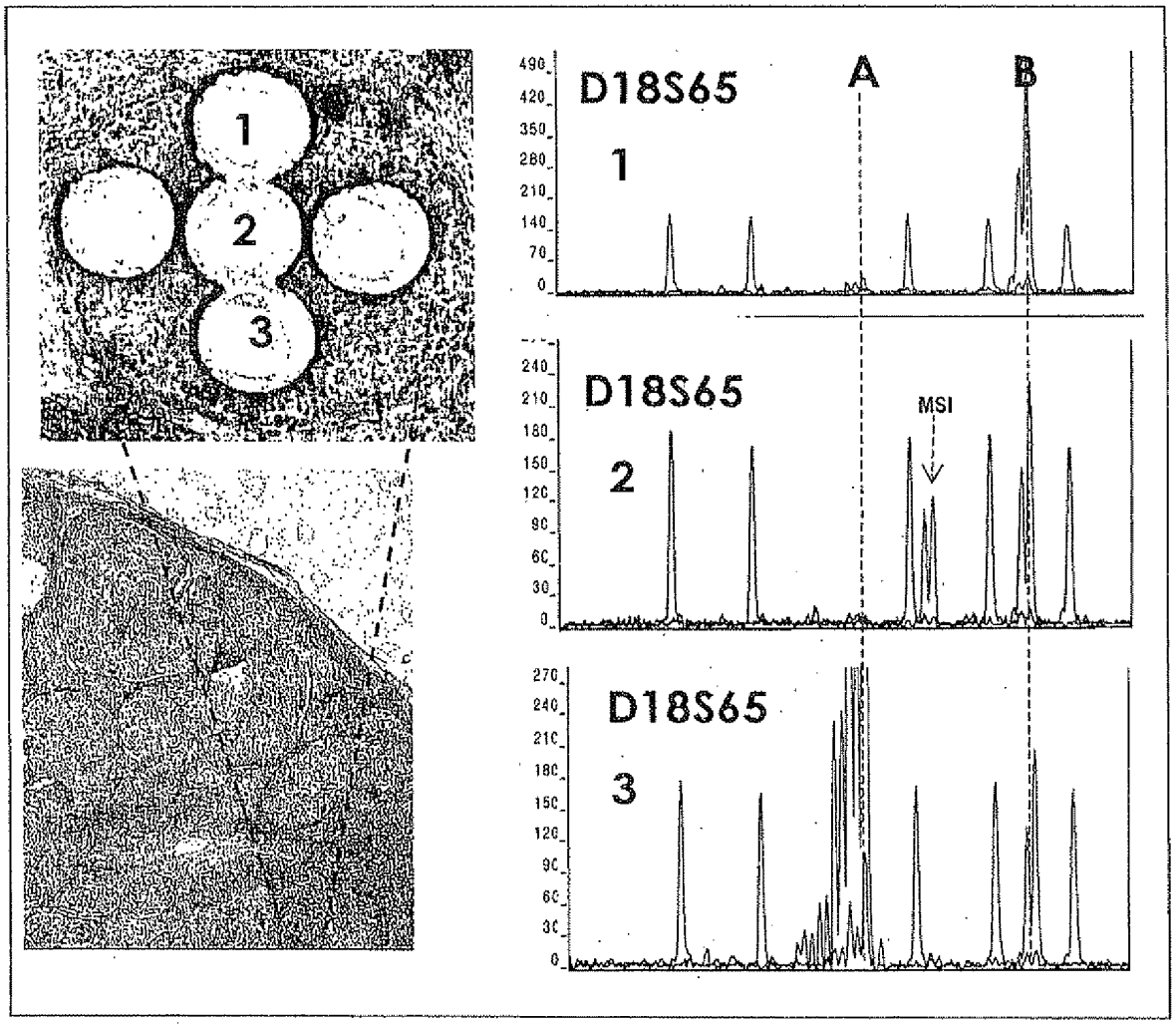


Figure 2.

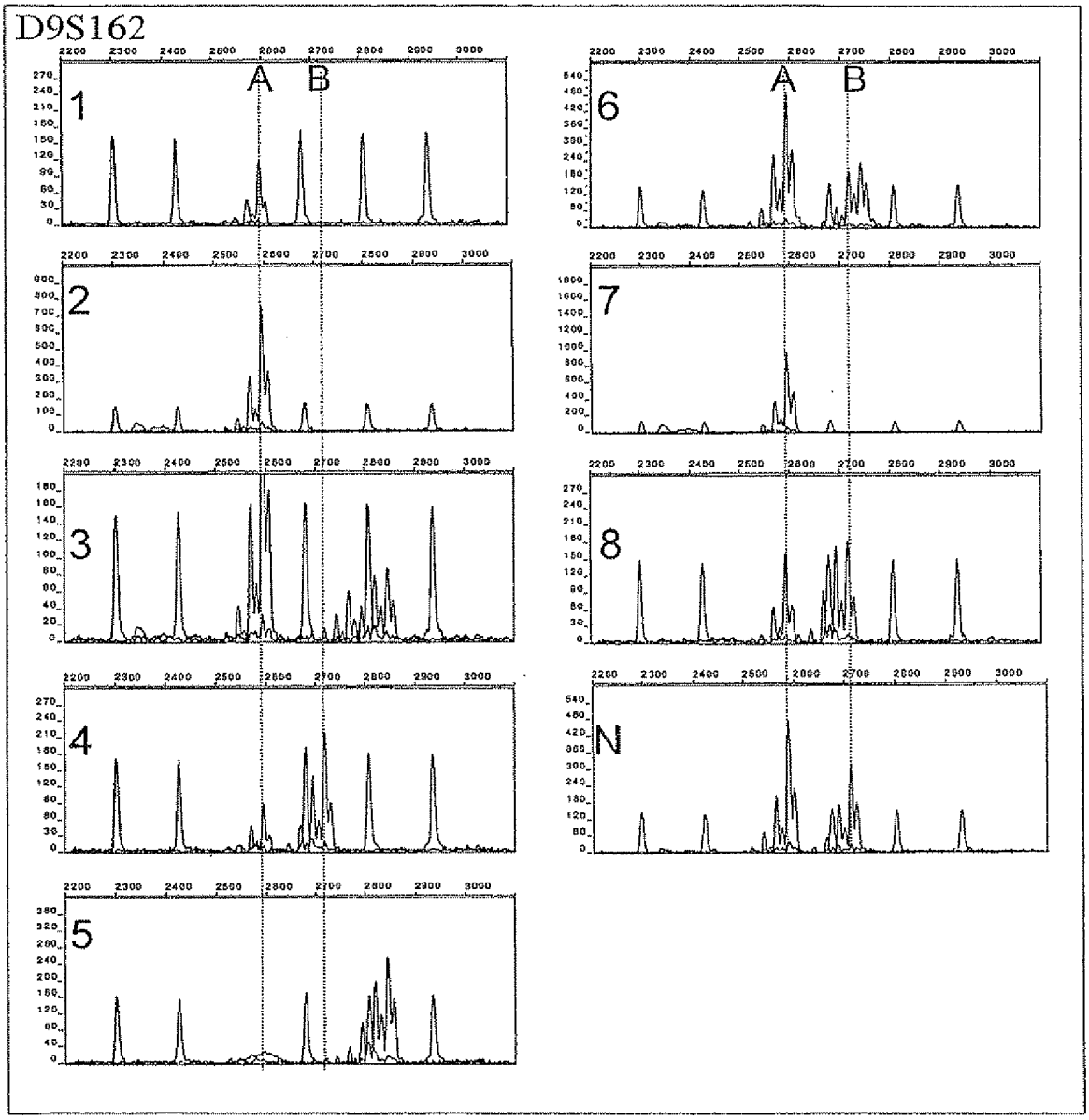


Figure 3.

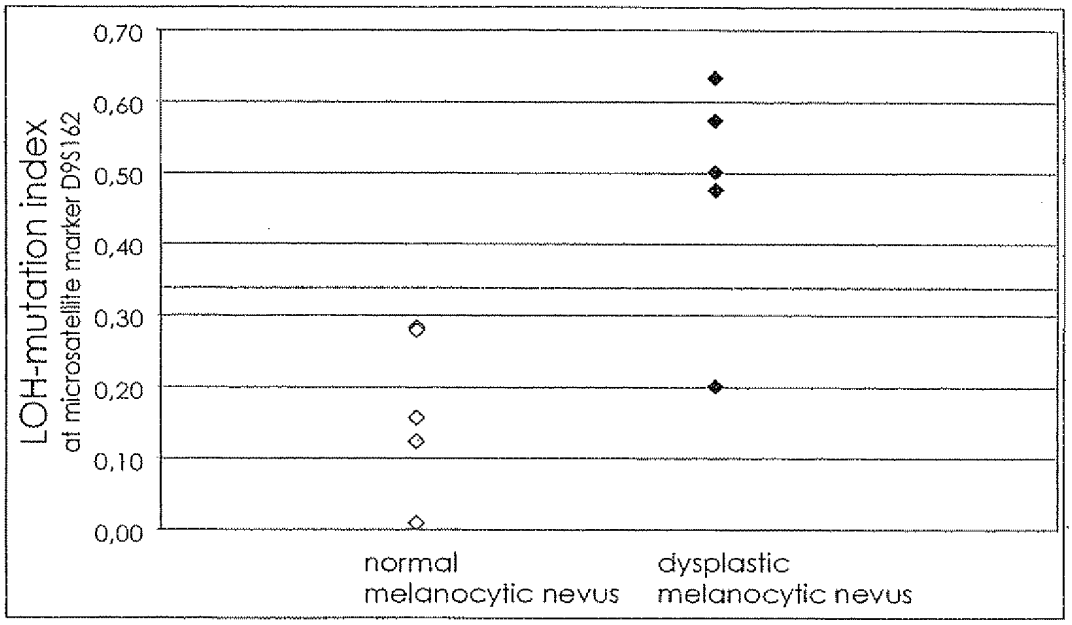


Figure 4.

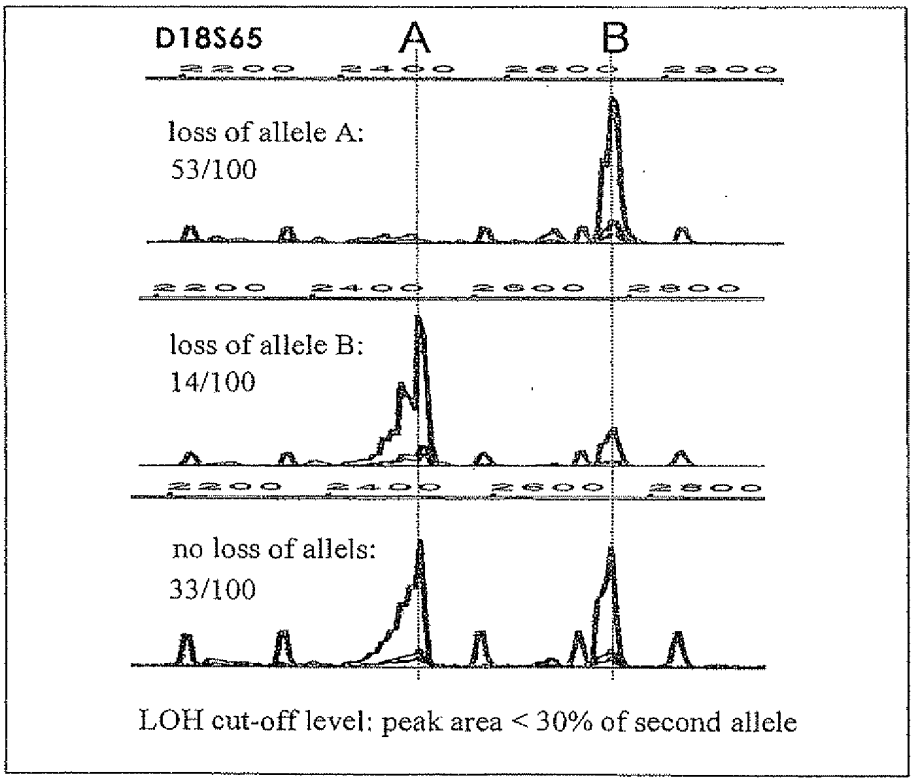


Figure 5. 4/5

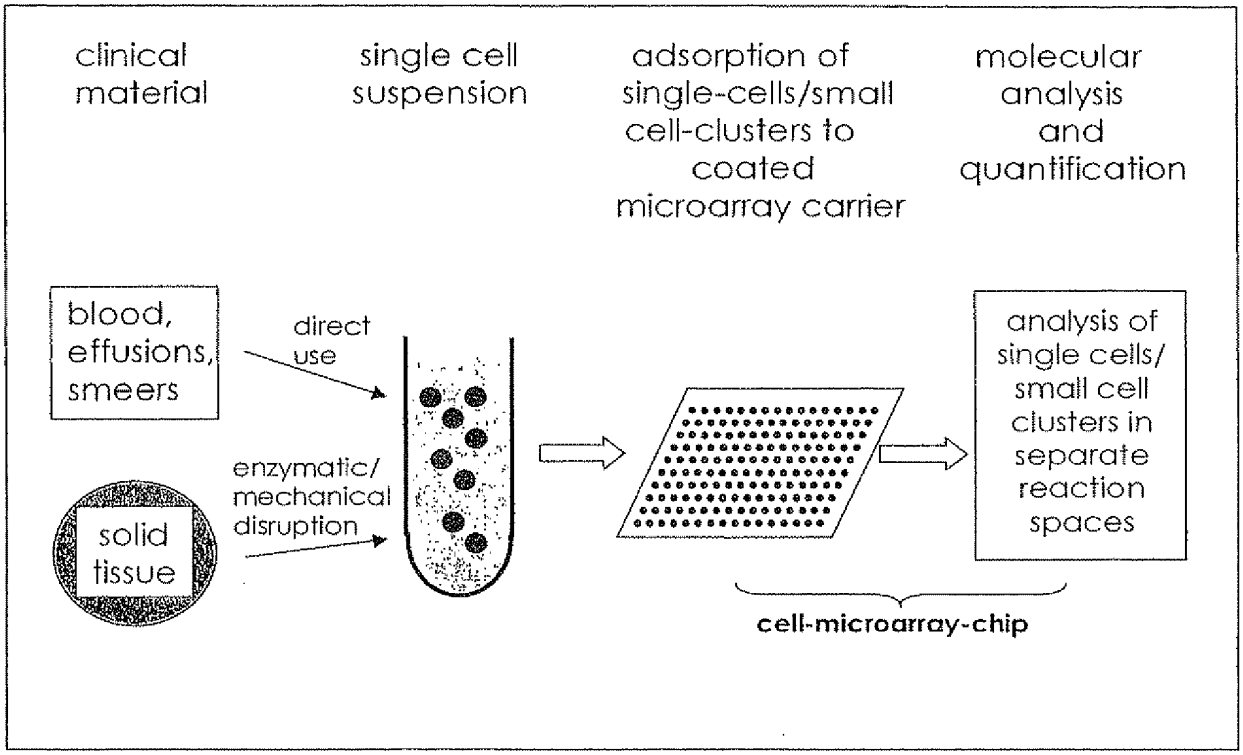


Figure 6.

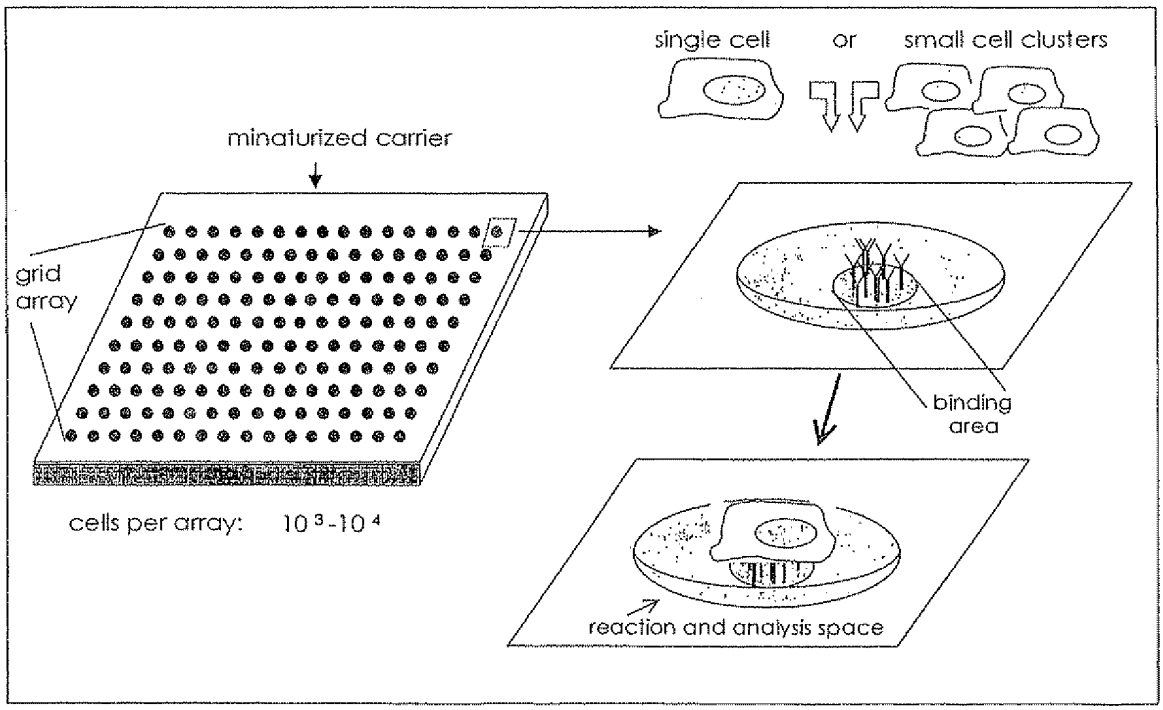


Figure 7.

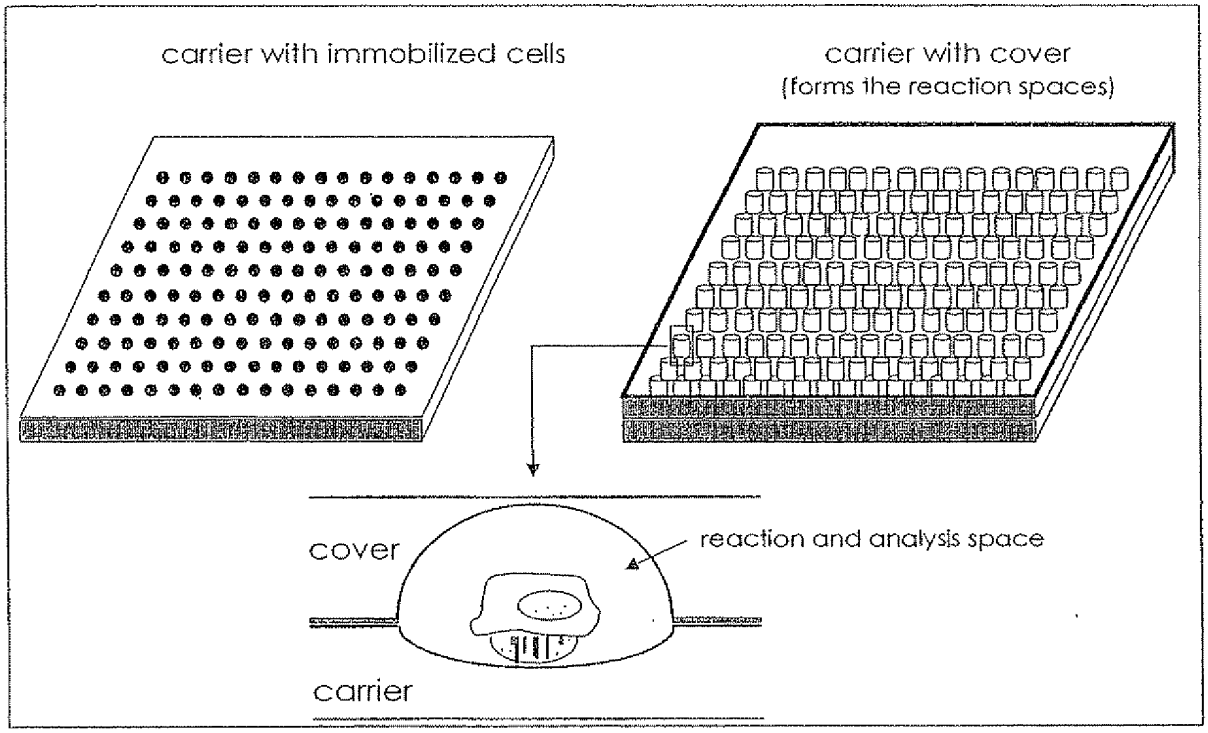
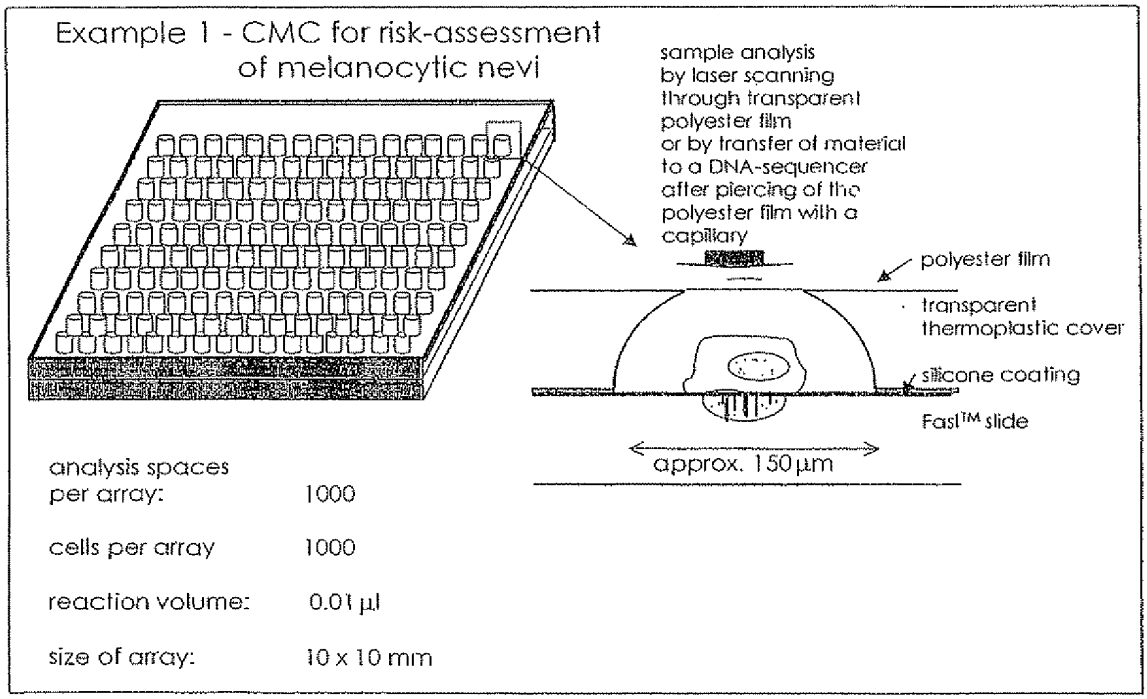


Figure 8.



INTERNATIONAL SEARCH REPORT

Inter Application No
PCT/IB 01/00696

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KLEIN ET AL: "COMPARATIVE GENOMIC HYBRIDIZATION, LOSS OF HETEROZYGOSITY, AND DNA SEQUENCE ANALYSIS OF SINGLE CELLS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 96, April 1999 (1999-04), pages 4494-4499, XP002144872 ISSN: 0027-8424 the whole document ---	1-8, 10-15,35
X	WO 00 17390 A (KLEIN CHRISTOPH ;MICROMET GES FUER BIOMEDIZINIS (DE); SCHMIDT KITT) 30 March 2000 (2000-03-30) the whole document ---	1-8, 10-15,35

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2002	07/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hagenmaier, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relavant passages	Relevant to claim No.
X	WALCH AXEL ET AL: "Microdissection of tissue sections: Application to the molecular genetic characterisation of premalignant lesions." PATHOBIOLOGY, vol. 68, no. 1, January 2000 (2000-01), pages 9-17, XP002188837 ISSN: 1015-2008 the whole document	1-8, 10-15,35
X	FEND F ET AL: "LASER CAPTURE MICRODISSECTION IN PATHOLOGY" JOURNAL OF CLINICAL PATHOLOGY, LONDON, GB, vol. 53, no. 9, September 2000 (2000-09), pages 666-672, XP001008052 ISSN: 0021-9746 the whole document	1-7, 11-15,35
Y	RÜBBEN ET AL.: "Analysis of tumor cell evolution in a melanoma: evidence of mutational and selective pressure fro loss of p16ink4 and for microsatellite instability" J.INVEST.DERMAT., vol. 114, no. 1, January 2000 (2000-01), pages 14-20, XP001053009 the whole document	8-10
Y	RÜBBEN ET AL.: "Analysis of LOH (9p,14q)-mutant fractions in dyaplastic melanocyte naevi and malignant melanomas reveals genetic instability as driving force of melanoma genesis" ARCHIVES OF DERMAT. RESEARCH, vol. 293, no. 1-2, February 2001 (2001-02), page 95/P208 XP001053013 the whole document	8-10
Y	BOLAND ET AL.: "Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression" NATURE MEDICINE, vol. 1, no. 9, September 1995 (1995-09), pages 902-909, XP002188838 the whole document	8,10
X	WO 00 60356 A (KAPUR RAVI ;ADAMS TERRI (US); CELLOMICS INC (US)) 12 October 2000 (2000-10-12) the whole document	14-35

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 84 03151 A (CHANG ET AL.) August 1984 (1984-08) the whole document	14-35
X	WO 88 08538 A (TANOX BIOSYSTEMS INC) 3 November 1988 (1988-11-03) the whole document	14-35
X	US 5 776 748 A (WHITESIDES GEORGE M ET AL) 7 July 1998 (1998-07-07) the whole document	14-35
X	CHRISTOPHERSON RICHARD I ET AL: "Immunophenotyping leukemias using a CD antibody microarray." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL, vol. 42, March 2001 (2001-03), page 159 XP001053227 92nd Annual Meeting of the American Association for Cancer Research;New Orleans, LA, USA; March 24-28, 2001, March, 2001 ISSN: 0197-016X the whole document	14-35
Y	US 5 985 658 A (COLINAS ROBERT JAMES ET AL) 16 November 1999 (1999-11-16) the whole document	14-35
Y	STILLMAN BRETT A ET AL: "FASTTM slides: A novel surface for microarrays." BIOTECHNIQUES, vol. 29, no. 3, September 2000 (2000-09), pages 630-635, XP002188847 ISSN: 0736-6205 the whole document	14-35
Y	WO 00 04382 A (ZYOMYX INC) 27 January 2000 (2000-01-27) the whole document	14-35
Y	WO 00 24940 A (US HEALTH ;VYSIS INC (US)) 4 May 2000 (2000-05-04) the whole document	14-35
A	WO 99 05321 A (TABONE JOHN C ;RAPIGENE INC (US); MOYNIHAN KRISTEN (US); NESS JEFF) 4 February 1999 (1999-02-04) the whole document	
A	WO 98 47003 A (KALININA OLGA V ;SILVER JONATHAN E (US); BROWN JAMES F (US); US HE) 22 October 1998 (1998-10-22) the whole document	

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INTERNATIONAL SEARCH REPORT

Inter Application No
PCT/IB 01/00696

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
E	WO 01 84150 A (BIOREF GMBH ;STAAB HANS JUERGEN (DE)) 8 November 2001 (2001-11-08) the whole document	14-35

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter Application No
PCT/IB 01/00696

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0017390	A	30-03-2000	AU	6192199 A	10-04-2000
			WO	0017390 A1	30-03-2000
			EP	1109938 A1	27-06-2001
			NO	20011316 A	18-05-2001
WO 0060356	A	12-10-2000	AU	4063700 A	23-10-2000
			EP	1166116 A1	02-01-2002
			WO	0060356 A1	12-10-2000
WO 8403151	A	16-08-1984	US	4591570 A	27-05-1986
			AT	77699 T	15-07-1992
			DE	3485785 D1	30-07-1992
			DE	3485785 T2	24-12-1992
			EP	0135541 A1	03-04-1985
			JP	60500732 T	16-05-1985
			WO	8403151 A1	16-08-1984
WO 8808538	A	03-11-1988	US	5100777 A	31-03-1992
			WO	8808538 A1	03-11-1988
US 5776748	A	07-07-1998	US	5976826 A	02-11-1999
US 5985658	A	16-11-1999	NONE		
WO 0004382	A	27-01-2000	AU	5102399 A	07-02-2000
			AU	5102599 A	07-02-2000
			EP	1097377 A2	09-05-2001
			EP	1097380 A1	09-05-2001
			WO	0004389 A2	27-01-2000
			WO	0004382 A1	27-01-2000
			US	6329209 B1	11-12-2001
WO 0024940	A	04-05-2000	AU	2973599 A	15-09-1999
			EP	1066517 A1	10-01-2001
			EP	1131470 A1	12-09-2001
			WO	9944062 A1	02-09-1999
			WO	0024940 A1	04-05-2000
WO 9905321	A	04-02-1999	AU	729134 B2	25-01-2001
			AU	8503298 A	16-02-1999
			CN	1265156 T	30-08-2000
			EP	1000175 A1	17-05-2000
			HU	0002356 A2	28-11-2000
			JP	2001511361 T	14-08-2001
			WO	9905321 A1	04-02-1999
			US	6248521 B1	19-06-2001
WO 9847003	A	22-10-1998	US	6143496 A	07-11-2000
			AU	7128998 A	11-11-1998
			WO	9847003 A1	22-10-1998
WO 0184150	A	08-11-2001	DE	10020704 A1	08-11-2001
			WO	0184150 A1	08-11-2001